UNCLASSIFIED UNLIMITED

DRES

A23172

CONTRACT REPORT 1/90

IMMUNOLOGIC CONSEQUENCES OF CHRONIC ADMINISTRATION OF PYRIDOSTIGMINE

R.J. Markham

Atlantic Veterinary College Charlottetown

May 1989

DEFENCE RESEARCH ESTABLISHMENT SUFFIELD, RALSTON, ALBERTA

"This work was carried out for DRES under contract. The accuracy of the information presented herein is the responsibility solely of the contractor and is NOT to be construed as an Official Department of National Defence position unless so designated by other authorizing documents."

WARNING

The use of this information is permitted subject to recognition of proprietary and patent rights.

UNCLASSIFIED

FINAL REPORT

IMMUNOLOGIC CONSEQUENCES OF CHRONIC ADMINISTRATION OF PYRIDOSTIGMINE

PRESENTED TO

Dr. J.G. Clement
Defence Research Establishment Suffield

BY

R.J.F. Markham
Atlantic Veterinary College
Charlottetown, P.E.I.

IMMUNOLOGIC CONSEQUENCES OF CHRONIC ADMINISTRATION OF PYRIDOSTIGMINE

CONTRACT 01SG-W7702-6-2247

Presented to
Dr. J.G. Clement
Defence Research Establishment Suffield

By
R.J.F. Markham
Atlantic Veterinary College
Charlottetown, P.E.I.

SUMMARY

The effect of subchronic adminstration of pyridostigmine on the immune system was evaluated in rats. Three aspects of the immune reactivity and host defence were measured: humoral immunity (antibody or B-lymphocyte-mediated; Jerne plaque assay and antibody accumulation), cell mediated immunity (T-lymphocyte mediated; blastogenesis and delayed skin reaction) and non-specific resistance (macrophage or NK cell activity; chemiluminescence). Pyridostigmine was administered by Alzet osmotic minipump to produce a 45-48% reduction in serum cholinesterase. There were no significant differences in any of the parameters that were measured among the control groups of rats or those treated with pyridostigmine. The measurement of body weights, organ weights, hemoglobin, RBC and WBC numbers as well as WBC differentials confirmed the apparent lack of effect of pyridostigmine on immune responsiveness. As the measurement included in this study had the capacity to measure most aspects of immune response and phagocytic function, it can be said with some confidence that the chronic administration of pyridostigmine, at the doses administered in this study, did not have any effect on immunological responsiveness.

INTRODUCTION

The discipline of immunotoxicology has grown immensely over the last numbers of years with the realization that exposure to environmental or therapeutic chemicals can have a significant influence on the immune response. This field of research now complements traditional toxicologic and pathologic studies of the effect of exogenous compounds on human or animal well-being.

The immune response and host defence mechanisms in general are highly orchestrated, highly organized systems involving the participation of a number of body systems. Alteration or inactivation of any of the components of the system can adversely affect the way the body responses to exogenous infectious agents or even endogenous processes like malignant transformation of host cells. The overall concern is that alteration of immune responsiveness will lead to decreased resistance to infectious disease, decreased resistance to development of tumours, decreased or adverse response to immunization or development of autoimmune disease. It is hoped that monitoring these chemicals for their effect on the immune response will prevent these conditions from developing.

Many tests are available to measure the immune response and host defence. These range from simple intradermal injection to elaborate culture techniques using sophisticated equipment (1-3). There is considerable difference in opinion about the usefulness of the individual tests, but it is generally agreed that there are three areas or three aspects of immune reactivity and host defence that must be measured: humoral immunity (antibody or B lymphocyte-mediated), cell mediated immunity (T lymphocyte- mediated) and non specific resistance (macrophage or NK cell activity). It appears necessary to measure all of these aspects because environmental or therapeutic chemicals do not necessarily have an universal effect on the host. Macrophage activity may be affected by a particular drug whereas B lymphocyte and T lymphocyte function may be spared any deleterious effect (4). A complete immunotoxicologic examination would contain tests to measure elements of each of these aspects of host defence.

Non-specific immunity is measured by challenge of the whole organism with an infectious agent or tumour with the subsequent determination of the time to clinical signs, death or development of tumour. Non-specific immunity can also be determined by

measuring the functional capacity of individual cells involved in this type of host defence - i.e. macrophage, neutrophil, or natural killer cell. These functions include chemotaxis, phagocytosis, intracellular killing, oxidative metabolism and cellular cytotoxicity.

B-lymphocyte immunity involves production of antibodies. Often, measurement of antibodies which have accumulated in serum is used as an indication of this type of immunity. Alternatively, it is possible to directly enumerate the antibody producing cells within a given lymphoid organ such as the spleen. Counting total B cell populations or measuring their proliferative response to mitogenic stimulation have also been used.

T-lymphocyte mediated immunity is often measured by skin test reactivity to intradermally injected antigen. More recently tests have been devised to measure T cell function directly by quantitating the proliferative response to mitogenic stimulation, or production of lymphokines (interferon, interleukin 2) or specific cytotoxicity following antigenic or mitogenic stimulation.

There are a wide range of compounds which have been shown to influence the immune response (5-7). Organophosphate compounds, commonly those associated with pesticide residues, have been shown to alter the immune response (8). The therapeutic compound pyridostigmine can inhibit, in a reversible manner, acetylcholine esterase. Because it shares this capacity with these known immunosuppressive compounds, combined with the fact that pyridostigmine is an established pretreatment for nerve agent poisoning, it is imperative that it be assessed for potential immunotoxicologic effects.

MATERIALS AND METHODS

ANIMALS:

Male CD (SD)BR rats weighing 250 g were obtained from Charles Rivers Ltd., Canada. They were housed in groups of 5 in the laboratory animal facilities at the Atlantic Veterinary College and had free access to food and water. They were acclimatized for 1-2 weeks before experiments began.

PUMPS:

Osmotic pumps were obtained from Alzet Corporation, Palo Alto, CA and were designed to deliver .5 μl/hour (Model 2002). They were filled with 50 mg/ml of pyridostigmine in accordance with manufacturers instructions. This dose was calculated to give greater than 50% reduction in acetylcholine esterase activity of blood (9) An additional dose representing one half of this value (25 mg/ml) was also used. They were implanted subcutaneously at the back of the neck of the rats under halothane anaesthesia.

CHOLINESTERASE ACTIVITY:

Rats implanted with pumps were bled at the time of implantation and 7 and 14 days after implantation. Blood was collected by cardiac puncture under halothane anaesthesia and the serum obtained was assayed for cholinesterase activity using a commercially available assay from Sigma Chemical Company, St Louis. (Procedure 420)

BODY AND ORGAN WEIGHTS:

Groups of rats implanted with pumps were weighed at 0, 7 and 14 days post implantation and blood was collected by cardiac puncture during halothane anaesthesia. On day 14, the rats were killed by halothane overdose and the spleen, thymus liver and kidneys were removed and weighed. Blood that had been collected in EDTA was submitted to the Diagnostic Laboratory for determination of haemoglobin, red blood cell numbers, white blood cell numbers and white blood cell differential.

JERNE PLAQUE ASSAY:

Groups of five rats were implanted with pumps containing 50 mg/ml pyridostigmine (PYR 50), 25 mg/ml pyridostigmine (PYR 25) or normal sterile saline. On day 5 and day 10 post implantation, groups of rats were immunized intraperitoneally with 0.75 ml of a 10% suspension of washed sheep red blood cells (SRBC). Four days following immunization, the rats were killed by an overdose of halothane and spleen was removed. A single cell suspension of the spleen was obtained by placing the spleen in a 60 mm petri dish containing 5 ml of medium RPMI and then by passing the tissue through a 80 mesh screen using a syringe plunger to gently force the tissue through the screen. This suspension was then passed through a syringe barrel containing cotton gauze to remove any large debris that may have been present. The filtered spleen suspension was then diluted in RPMI prior to use.

Poly-L-lysine (PLL) (Sigma, molecular weight > 400,000) was diluted to 0.06 mg/ml in phosphate buffered saline (PBS). Two hundred and fifty millilitres of the PLL was dispensed into each of the wells of a 24-well plastic culture plate (Falcon 3047). These plates were allowed to stand at room temperature for 15-20 minutes and were then washed twice with PBS. A 2% suspension of washed sheep red blood cells (SRBC) was made in Hank's Balanced Salt Solution (HBSS) and 0.5 ml of this suspension was added to the wells which had previously been coated with PLL. The SRBC were incubated for fifteen minutes, the plates were gently agitated to resuspend the SRBC in the wells and incubation continued for another fifteen minutes. The plates were then washed by immersion in saline, and the wells were filled by inversion of the plates in the saline bath. The plates were washed for 15 minutes, emptied by tipping and gentle shaking and then filled with 250 μl of medium consisting of 12% heat inactivated (56°C, 30 min) fetal bovine serum (FBS) in RPMI containing 5% guinea pig serum (Gibco Labs) as a source of complement. Diluted aliquots of spleen cells were then added in a volume of 250 μl and the mixture was incubated at 37°C with 5% CO_2 for 1 hour. After incubation, cells were fixed for 10 minutes by addition of 50 μl of 10 % gluteraldehyde to each well and rinsed with water and allowed to dry. The wells of the plates were examined under low power microscopy to detect the presence of plaques (clear areas) in the SRBC monolayer in the wells. These

plaques were counted and expressed as the number of plaques per million spleen cells.

The in vitro Jerne plaque assay was performed as described by Rodgers et al (10). Spleen cell suspensions were made from a normal or drug treated rats and were diluted to a concentration of 2 x 10^7 cells/ml of medium RPMI containing 20 % FBS, 15mM Hepes buffer, antibiotics and 5 x 10^{-5} B mercaptoethanol. One half milliliter of this suspension was dispensed into the wells of a 24-well culture plate. Thirty microliters of a 1% suspension of SRBC was added to each well and the cultures were incubated with shaking for 4 days at 37°C in a 5% CO_2 atmosphere. After incubation, cells were retrieved from the wells and used in the Jerne Plaque assay described above.

BLASTOGENESIS:

The procedures for in vitro and in vivo blastogenesis followed the same basic protocol.

Spleen cell suspensions were obtained as described for the Jerne Plaque assay except that the medium used was HBSS without calcium and magnesium and the final volume of the suspension was ten milliliters. This suspension was placed on 6 ml of Ficoll/Paque (Pharmacia) in a 120x15 mm glass tube and was centrifuged at 500 x G for 20 minutes at room temperature. Mononuclear cells were collected from the Ficoll/Paque-media interface, washed twice with HBSS and resuspended in RPMI containing 10 % heat inactivated fetal bovine serum, 100 U penicillin/ml and 100 μg streptomycin/ml. Cells were counted and resuspended to 1.0 x 10^6 /ml and 200 μl were dispensed into the wells of 96 well microtiter culture plates (Falcon, 3072). To some cultures, concanavalin A (CONA) was added to give a concentration of 10, 25, 50 or 100 μg/ml cell suspension. Other wells remained as unstimulated controls. After 3 days culture at 37°C in a 5% CO_2 incubator, tritiated thymidine (2 μCi) (ICN) was added to each well in a volume of 2 μl and incubation continued for an additional 24 hours. These cell cultures were harvested immediately or placed at 4°C to stop the reaction and harvested at a latter date. Harvesting was performed by collection of the cells from the microtiter plates using an automated cell harvester (Skatron) with deposition onto glass fibre filter discs. The discs were then dried, placed into scintillation vials and after addition of the fluor (Beckman, Ready-Safe), radioactivity was determined in a scintillation counter (Beckman LS 1701) expressing the activity as counts per minute.

For in vivo blastogenesis, spleens from animals implanted with drug or saline

containing pumps were taken on days 7 and 14 post implantation and cells from these spleens were tested for their proliferative response to the mitogen (CONA) as described above.

For in vitro blastogenesis, spleens from normal rats, not implanted with pumps were taken and cells placed into culture. To some of the cultures in the microtiter plates, pyridostigmine was added at a molar concentration of 10^{-3} M to 10^{-9} M prior to addition of the CONA. The drug was present throughout the entire incubation period. Cell cultures without CONA served as controls.

An alternative approach to measure cell proliferation in response to CONA was also employed. This method relies on alteration of a tetrazolium salt by living but not dead cells and utilizes a colorimetric detection of this activity. The techniques has been described by Mossman (11). Lymphocyte cultures were established as described above. After incubation with mitogen for three days, 10 μl of a 5 mg/ml solution of MMT (3-(4,5 methylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) were added to each well and the cultures were incubated at 37°C for 4 hr. One hundred microliters of acetone/isopropanol (1:3 v/v) were added to each well and incubated a room temperature for 10-15 minutes to ensure that all the dark blue crystals had dissolved. The microtiter plate was then read in an automated ELISA reader using a test wavelength of 570 nm and a reference wavelength of 630 nm.

CHEMILUMINESCENCE:

Both the in vitro and in vivo tests for chemiluminescence used the same basic protocol. Rats were injected intraperitoneally with 10 ml of thioglycollate broth (29 gm/l) to encourage accumulation of cells in the peritoneum. Five days after injection, cells were harvested from rats killed with halothane by peritoneal lavage using HBSS without calcium or magnesium. The cells were washed twice in the same medium and finally resuspended to 1.0 or 2.5 x 10^6 cell/ ml of RPMI + 5% FBS. Four hundred microliters was dispensed into scintillation vials. To induce chemiluminescence, a phagocytosible particle, zymosan, was used. Zymosan (5mg/ml saline) was boiled for 30 minutes and washed twice by centrifugation at 700 xG. The pellet was mixed and incubated with 5 ml of fresh rat serum at 37°C for 30 minutes. The mixture was centrifuged and the supernatant fluid extracted. The pellet, containing the zymosan was then diluted to 5 mg/ml in HBSS and stored at -

80°C. In the chemiluminescence assay, this stock solution was diluted to 0.5 mg/ml with RPMI + FBS and 200 μl were added to the vials containing the cells. Thirty microliters of 2 x 10^{-3} M lucigenin was then added to the vials. The vials were then incubated for 10-15 minutes at 37°C and then placed in a scintillation counter in an out-of-coincidence mode (Packard 2000CA). Data was expressed as CPM. Cultures without zymosan served as controls.

For in vitro assays, cells were collected from normal rats that had been injected with thioglycollate and cultures established with the peritoneal lavage cells were incubated for 1 hour with 10^{-3} M TO 10^{-9} M pyridostigmine prior to addition of the zymosan and the lucigenin.

For in vivo chemiluminescence, cells collected from groups of rats implanted with pumps were assayed at 7 and 14 days post implantation having been injected with thioglycollate 5 days prior to collection of peritoneal cells.

SKIN TEST:

Two techniques were utilized to assess skin test reactivity in rats. Groups of rats implanted with pumps were immunized with 1 mg of keyhole limpet haemocyanin (KLH) or 0.75 ml of 10 % SRBC 6 days after the pumps had been implanted. Blood was collected and assayed by ELISA or haemagglutination (see below) to monitor success of immunization. At 45 days post immunization, the hair on the back of the neck of the rats was removed and sites on the skin marked. Skin thickness was determined using double skin fold thickness measurement using callipers. These sites were then injected intradermally with 0.1 ml saline or 0.1 of a 2% suspension of SRBC or 0.1 ml of a 500 μg/ml KLH. Skin thickness was monitored at 8, 24, 48 and 72 hours after injection.

Alternatively, rats that had been implanted with pumps were injected intradermally with 0.1 ml of a 4000 μg/ml solution of phytohaemagglutinin without any prior sensitization. Double skin fold thickness was monitored before and after injection.

HAEMAGGLUTINATION AND ELISA:

Antibody response to SRBC and KLH were measured by haemagglutination and enzyme immunosorbent assay (ELISA) respectively. For haemagglutination, 50 μl of serum was added to 50 μl of saline in 96-well haemagglutination trays and doubling dilutions of

the serum was made. Fifty microliters of a 2 % suspension of SRBC was added to each well and the plate was gently shaken. Incubation at room temperature continued for 2-3 hours. The titer of the serum was given as the last dilution showing haemagglutination.

For the ELISA, 5 μg KLH in 100 μl carbonate buffer (pH 9.6, 0.1M) was placed in the wells of microtiter plates and incubated at room temperature for 18 hours. The plates were washed, blocked and serum diluted and conjugate and substrate added (see appendix A). Colour development was recorded on an automated ELISA recorder (BioTek EL310). Titer was determined as that dilution of serum giving an absorbance reading of greater than 0.1.

STATISTICAL ANALYSIS OF DATA:

Data from single trials involving 5 animals or repeats of trials in which data had been pooled were analyzed by analysis of variance.

RESULTS

ACETYLCHOLINESTERASE:

Rats implanted with osmotic pumps containing 50 mg/ml pyridostigmine had serum cholinesterase activity which represented a 45-48 % reduction when compared to levels present in serum prior to implantation of the pumps.

BODY AND ORGAN WEIGHTS

All animals gained weight during the course of the two week time period (TABLE 1) with percentage gain being 20, 22 and 19 for the saline, 25 PYR and 50 PYR groups, respectively. There was no significant difference in the weights of the animals at each of the times when they were weighed.

Although there appeared to be a decrease in the weight of the thymus from rats treated with PYR 50, this difference was not statistically significant. Likewise, there were no significant difference between the three groups of rats when the weights of the spleen, liver

and kidneys were taken 14 days after pumps had been implanted. (TABLE 2).

HAEMATOLOGY:

Haematologic data are presented in TABLES 3 and 4. Differences in haemoglobin or red and white blood cell numbers could not be found when these parameters were compared among treatment groups prior to and 7 and 14 days after implantation of the pumps. There was an apparent decrease in lymphocyte numbers in the saline and PYR 50 group on day 14 but these differences were not significant.

PLAQUE FORMING CELLS:

The response of rats to injection with SRBC is given in TABLE 5. A mean number of approximately 800 plaque-forming cells/10^6 spleen cells was obtained for all three groups of rats. There was no significant difference among response of rats which had obtained saline pumps or osmotic pumps containing the two doses of drug. This was evident at both 7 days and 14 days post implantation.

The in vitro plaque forming cell assay failed to demonstrate any plaques despite several attempts to establish this assay.

SKIN TEST AND SERUM ANTIBODY TITERS.

Rats immunized with either SRBC or KLH at 6 days after implantation of the pumps responded well in terms of specific serum antibody (TABLES 6 and 7). No significant difference in titers could be demonstrated between the control saline group or the two drug treated groups.

These rats, however, did not respond well to intradermal injection of the antigen in an attempt to demonstrate specific skin test reactivity. Rats immunized with SRBC and challenged intradermally with SRBC failed to show any significant difference between thickness of injection sites receiving antigen and those receiving saline as control. Among rats immunized with KLH, only rats from the group receiving 50 mg/ml pyridostigmine in their pumps showed differences between thickness of skin between antigen and control saline sites. Overall, no differences were present between the three treatment groups.

Rats, however, implanted with pumps and given intradermal injections of PHA did give

a good skin test response to this mitogen (TABLE 8). The most intense reaction took place at 8 hours post-injection and continued at 24 hours post injection. Although there was an apparent decrease in the drug treated rats, particularly in the PYR 25 group, this difference was not significant. The values for rats injected with PHA represent skin thickness from which control saline injection site thickness had been subtracted.

BLASTOGENESIS:

The mitogenic response of spleen cells from rats treated with osmotic pumps loaded with saline or drug is given in Table 9. The data is expressed as the mean log CPM of cultures stimulated with optimal concentration of CONA which in all cases was either 25 or 50 mg/ml. There was no significant difference in the ability of the spleen cells from rats from the three treatment groups to proliferate upon exposure to CONA. Similarly, spleen cells from these same groups showed no difference in their basal rate of multiplication in culture (cells alone from TABLE 9). Spleen cells derived from normal rats and treated in culture with pyridostigmine, responded in a similar manner as control cells, both in terms of basal rats and proliferation induced by CONA (TABLE 10). Differences apparent in this are not statistically significant.

It was not possible to utilize the colorimetric test to measure cell proliferation as performed under conditions described in this text.

CHEMILUMINESCENCE:

Peritoneal exudate cells derived from rats implanted with pumps responded similarly to stimulation with zymosan. This was apparent at both cell concentration used for the assay. No significant differences could be found among the three treatment groups (TABLE 11).

Peritoneal exudate cells elicited from normal rats were not influenced by the presence of pyridostigmine in the culture during stimulation with zymosan. No significant differences could be detected between the three treatment groups (TABLE 12).

DISCUSSION

There were no significant differences in any of the parameters that were measured among the control groups of rats or rats treated with pyridostigmine. As the measurements included in this study had the capacity to measure most aspects of immune responsiveness and phagocytic function, it can be said with some confidence that chronic administration of pyridostigmine, at the doses given in this study, did not have any effect on immunological responsiveness.

The ability to produce specific antibodies was measured in two different manners. The Jerne plaque technique measures the number of lymphocytes producing antibody shortly after administration of antigen i.e. during the induction phase. This test was technically easy to perform and requires minimal sophisticated equipment and has become an established method for determining humoral response. In the rat, it was noted that substantial variation in the response had occurred and so sample size and transformation of the data was utilized to obtain as normal distribution of the values. Humoral immunity was also detected by measuring the accumulation of antibodies in serum over a period of time. Again these tests are simply performed although the ELISA test does require an automated reader to make it a practical test.

There were several tests devised for determination of cell mediated immunity. The lymphocyte transformation test (blastogenesis) measures the proliferative response of lymphocytes to a variety of phytomitogens and bacterial products. The response to the mitogens PHA and CONA is thought to reflect T- lymphocyte activity. This test was employed both as an in vivo test using splenic lymphocytes from drug treated rats and as an in vitro test where normal cells in culture are exposed to the drug at the time of or shortly before stimulation with mitogen. The opportunity to utilize the same test for both in vitro and in vivo studies can simplify and broaden the capacity to measure immunological responsiveness. The most common method to detect proliferation the T lymphocytes upon stimulation by mitogen is to provide a radiolabelled nucleic acid precursor such as thymidine with the amount of cell-associated radioactivity being proportional to the degree of proliferation. It has been reported that colorimetric tests are also available which can

detect viable cells and increases in viable cell upon proliferation. These tests are attractive in that they avoid the use of radioisotopes which commonly have very long half-lives (i.e. tritiated thymidine). This test, however, as performed in the protocol listed above, fell far short of the sensitivity obtained by the use tritiated thymidine despite numerous modifications and thus cannot be recommended at this time.

An additional test which has been used for many years to measure cell-mediated immunity in vivo is the delayed skin test reaction. This measures the ability of T lymphocytes to promote accumulation of cells in the area of an intradermal injection of antigen. Utilizing the immunization schedule given in this report, it was not possible to demonstrate a strong skin test reaction to either a particulate antigen (SRBC) or a soluble protein (KLH). Our immunization regimen did not include potent adjuvants such as Freund's Complete Adjuvant. This material produces significant lesions in animals with subsequent pain and suffering and its use has been limited by the Canada Council on Animal Care and will likely see further reductions in its use. Other adjuvants will have to be developed to eventually replace this material. There have been several reports in a variety of animal species that phytomitogens like PHA or CONA when injected intradermally can mimic the skin test reactivity produced in animals challenged with specific antigens (12-14). Rats challenged with the mitogen PHA responded with a substantial increase in skin thickness. Histologic examination of the reaction revealed the presence of intradermal edema and the accumulation of many inflammatory cells including mononuclear cells, however, with a predominance of polymorphonuclear cells. This is similar to some reports on the use of PHA in other species. While the classic delayed skin test is thought to consist mainly of mononuclear cells, there is a substantial involvement of neutrophils, particularly early in the response. While the intradermal injection of PHA may be measuring some non-specific (non- T lymphocyte-mediated) inflammation, it is simple to perform and appears to be a good measure of the host's ability to mobilize inflammatory cells.

The measurement of chemiluminescence indicates the capacity of phagocytic cells to undergo and oxidative metabolic burst during phagocytosis or in response to soluble material which can trigger this response. This oxidative burst is an important mechanism by which phagocytes deal with invading bacteria, protozoa etc and deficiencies in this

capacity can significantly jeopardize host defence mechanisms. This test was performed on cells elicited by the injection of an irritant into the peritoneal cavity. The cells accumulating by 5 days were predominantly mononuclear in nature with morphological characteristics of macrophages. When stimulated with yeast cell walls (zymosan) oxidative metabolism was stimulated in these macrophages and the products of this burst in the presence of the chemical lucigenin emitted chemiluminescence which can be detected as photons of energy in a standard scintillation counter (in the out of coincidence mode) or specialized apparatus designed specifically for measuring chemiluminescence. Again, this test was used to measure the effect of drug administered in vivo and well as on cells cultured in vitro and exposed to drug prior to addition of zymosan.

The measurement of body weights, organ weights, haemoglobin, RBC and WBC numbers as well as WBC differentials confirmed the apparent lack of effect of pyridostigmine on immune responsiveness.

This panel of test can be established in most modern immunology laboratories an provide a broad measure of the immune response and possible alterations of this response by environmental or therapeutic chemicals.

REFERENCES

1. Krakowka, S. In vitro and in vivo methods in immunotoxicology Toxicol Pathol 15:276 1987.

2. Koller, LD. Immunotoxicology today. Toxicol Pathol 15:346 1987.

3. Dean, JH., Thurmond, LM. Immunotoxicology: and overview. Toxicol Pathol 15:265 1987.

4. Allen, AC., Koller, LD., Pollack, GA. Effect of toxaphene exposure on immune responses in mice. J Toxicol Environ Health. 11:61 1982.

5. White, KL. An overview of immunotoxicology and carcinogenic polycyclic aromatic hydrocarbons. Envir Carcino Rev C4: 163 1986.

6. Fournier, M., Bernier, J., Flipo, D., Krzystyniak, K. Evaluation of pesticide effects on humoral immune response to sheep erythrocytes and mouse hepatitis virus 3 by immunosorbent analysis. Pesticide Biochem Physiol 26:353 1986.

7. Exon, JH., Kerkvliet, NI., Talcott, PA. Immunotoxicology of carcinogenic pesticides and related compounds. Environ Carcino Rev. C5:73 1987.

8. Rodgers, KE., Leung, N., Imamura, T., Devens, BH. Rapid in vitro screening assay for immunotoxic effects of organophosphorous and carbamate insecticides on the generation of cytotoxic T-lymphocyte responses. Pesticide Biochem and Physiol 26:292 1986.

9. Shiloff JD., Clement, JG. Effects of subchronic pyridostigmine pretreatment on the toxicity of soman. Can J Physiol Pharmacol 64: 1047 1986.

10. Rodgers, KE., Imamura, T., Devens, BH. Investigations into the mechanism of immmunosuppression caused by acute treatment with O,O,S-trimethyl phosphorothioate: generation of suppressive macrophages from treated animals. Toxicol appl Pharmacol 88:270 1987.

11. Mosmann, T. Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55 1983.

12. Wilkie, BN., Markham., RJF., Hazlett, C. Deficient cutaneous response to PHA-P in healthy puppies from a kennel with high prevalence of demodicosis. Can J Comp Med 43:415 1979.

13. Hodgin, EC, McGuire, TC., Perryman, LE., Grant, BD. Evaluation of delayed hypersensitivity responses in normal horses and immunodeficient foals. Am J Vet Res 39:1161 1978.

TABLE 1: Body Weight of Rats Following Implantation of Osmotic Pumps Containing Saline or Pyridostigmine

Treatment	Days Following Implantation		
	0	7	14
Saline (5)	305 ± 13*	336 ± 11	382 ± 24
25 mg/ml Pyridostigmine (5)	300 ± 11	336 ± 14	382 ± 15
50 mg/ml Pyridostigmine (5)	298 ± 14	331 ± 15	370 ± 24

* Mean weight in grams ± standard deviation
Numbers in parentheses indicate number of rats used

TABLE 2: Organ Weights of Rats Following Implantation of Osmotic Pumps Containing Saline or Pyridostigmine

Treatment	Organ Weights*			
	Spleen	Thymus	Liver	Kidney
Saline (5)	.85 ± .17	.62 ± .04	14.1 ± 1.29	3.03 ± .13
25 mg/ml Pyridostigmine (5)	.81 ± .11	.69 ± .07	15.2 ± 1.22	3.02 ± .24
50 mg/ml Pyridostigmine (5)	.84 ± .23	.55 ± .13	14.01 ± 1.26	3.02 ± .19

*Mean weight in grams ± standard deviation of organs taken 14 days after pump implantation
Numbers in parentheses indicate number of rats used.

TABLE 3: Haematologic Parameters of Rats following Implantation of Osmotic Pumps containing Saline or Pyridostigmine

Treatment	Days Following Implantation								
	0			7			14		
	Hgb*	RBC**	WBC+	Hgb	RBC	WBC	Hgb	RBC	WBC
Saline (10)	136.9 ± 3.8	6.6 ± .3	15.8 ± 3.7	136.9 ± 3.8	6.3 ± .2	16.6 ± 3.8	144.5 ± 2.1	6.7 ± .2	15.0 ± 6.6
25 mg/ml Pyr. (5)	141.4 ± 4.1	6.6 ± .3	12.6 ± 3.4	135.8 ± 3.4	6.2 ± .2	16.6 ± 3.2	146.0 ± 3.4	6.8 ± .3	15.0 ± 3.2
50 mg/ml Pyr. (10)	136.7 ± 4.2	6.5 ± .3	12.5 ± 2.5	138.0 ± 4.5	6.3 ± .3	16.4 ± 4.6	148.6 ± 5.2	6.9 ± .3	14.5 ± 8.8

* Mean Haemaglobin in g/L ± standard deviation

**Mean Red blood cell counts x 10^{12}/l ± standard deviation

+ Mean White blood cell counts x 10^{9}/l ± standard deviation

Numbers in parentheses indicate number of rats used

TABLE 4: White Blood Cell Differentials of Rats after Implantation of Osmotic Pumps Containing Saline or Pyridostigmine

Treatment	Days Following Implantation								
	0			7			14		
	L*	S	M	L	S	M	L	S	M
Saline (10)	81.9 ± 9.4	16.6 ± 8.8	0.7 ± 0.9	83.9 ± 7.9	14.6 ± 7.8	0.8 ± 1.4	73.3 ± 14.2	24.8 ± 13.8	0.6 ± 1.1
25 mg/ml Pyr. (5)	84.0 ± 6.9	15.6 ± 6.5	0.2 ± 0.5	86.0 ± 4.5	13.6 ± 4.8	0.6 ± 0.8	85.4 ± 5.6	13.6 ± 4.7	0.2 ± 0.7
50 mg/ml Pyr. (10)	86.9 ± 5.5	12.2 ± 5.3	0.56 ± 0.7	86.1 ± 7.2	12.9 ± 6.7	0	74.8 ± 12.9	24.1 ± 12.4	0.3 ± 0.5

* Mean percentages of lymphocytes (L), segmented neutrophils (S) or monocytes (M) ± standard deviation
Numbers in parentheses indicate number of rats used

TABLE 5: Plaque Forming Cell Response of Rats Immunized with SRBC after Implantation of Osmotic Pumps Containing Saline or Pyridostigmine

Treatment	Days Following Implantation	
	9	14
Saline	2.63 ± .51* (26)	2.81 ± .38 (13)
25 mg/ml Pyridostigmine	2.83 ± .25 (12)	2.72 ± .43 (17)
50 mg/ml Pyridostigmine	2.76 ± .41 (30)	2.77 ± .56 (17)

* Mean Log number of plaque forming cells / 10^6 spleen cells ± standard deviation
Numbers in parentheses indicate number of rats used

TABLE 6: Serum Haemagglutination Titers of Rats Immunized with SRBC after Implantation of Osmotic Pumps Containing Saline or Pyridostigmine

Treatment	Days Post Immunization*		
	0	14	28
Saline (5)	0**	9.2 ± .84	9.2 ± .84
25 mg/ml Pyridostigmine (5)	0	10 ± .71	10.2 ± .45
50 mg/ml Pyridostigmine (5)	0	10.2 ± .45	10.2 ± .45

* Rats were immunized 6 days after implantation of pumps

**Mean Log_2 titer ± standard deviation

Numbers in parentheses indicate number of rats used

TABLE 7: Serum ELISA Titers of Rats Immunized with KLH after Implantation of Osmotic Pumps Containing Saline or Pyridostigmine

Treatment	Days Post Immunization*		
	0	14	28
Saline (5)	1.32 ± 1.21**	2.78 ± .55	3.14 ± .54
25 mg/ml Pyridostigmine (5)	0	2.66 ± .54	3.08 ± .72
50 mg/ml Pyridostigmine (5)	0	3.14 ± .39	3.53 ± .72

* Rats were immunized 6 days after implantation of pumps

**Mean Log titer ± standard deviation

Numbers in parentheses indicate number of rats used

TABLE 8: Skin Test Reaction to Intradermal Injection of Phytohaemagglutination in Rats after Implantation of Osmotic Pumps Containing Saline or Pyridostigmine

Treatment	Days Following Implantation			
	7		14	
	8 hr.*	24 hr.	8 hr.*	24 hr.
Saline (12)	6.0 ± .8	4.2 ± .4	6.2 ± .7	5.6 ± .7
25 mg/ml Pyridostigmine (5)	5.2 ± .6	3.7 ± .7	5.5 ± .2	5.0 ± .1
50 mg/ml Pyridostigmine (12)	5.6 ± .7	3.9 ± .7	6.4 ± .9	5.7 ± .9

* Mean increase in double skin fold thickness (± standard deviation) at 8 hr. or 24 hr. after intradermal injection
Numbers in parentheses indicate number of rats used

TABLE 9: Blastogenic Response of Mononuclear Leukocytes from Rats after Implantation of Osmotic Pumps Containing Saline or Pyridostigmine

Treatment	Days Following Implantation			
	7		14	
	Cells Alone*	Cells + CONA*	Cells Alone	Cells + CONA
Saline	2.55 ± .32	4.13 ± .47 (22)	2.82 ± .35	4.24 ± .26 (23)
25 mg/ml Pyridostigmine	2.73 ± .29	4.39 ± .28 (23)	2.74 ± .43	4.22 ± .38 (24)
50 mg/ml Pyridostigmine	2.68 ± .26	4.39 ± .31 (23)	2.67 ± .28	4.21 ± .33 (24)

* Mean Log CPM in lymphocyte cultures ± standard deviation
Numbers in parentheses indicate number of rats used

TABLE 10: Blastogenic Response to CONA of Rat Spleen-derived Mononuclear Leukocytes Exposed to Pyridostigmine in vitro

Concentration of Pyridostigmine	CPM*	
	Cells Alone	25 μg CONA
0	2.74 ± .14 (12)	4.25 ± .21 (12)
10^{-3} M	2.56 ± .13 (13)	4.08 ± .30 (13)
10^{-5} M	2.58 ± .13 (13)	4.07 ± .24 (13)
10^{-7} M	2.78 ± .17 (13)	4.24 ± .20 (13)
10^{-9} M	2.76 ± .16 (12)	4.28 ± .19 (12)

*Mean Log CPM of control cell cultures and cell cultures stimulated with CONA
Numbers in parentheses indicate number of rats used

TABLE 11: Chemiluminescence Response of Peritoneal Lavage Cells from Rats after Implantation of Osmotic Pumps Containing Saline or Pyridostigmine

Treatment		Days Post Immunization	
	Cell Concentration*	7	14
Saline (5)	1.0×10^6	6.23 ± .06** (8)	6.30 ± .19 (8)
	2.5×10^6	6.55 ± .10 (8)	6.67 ± .10 (8)
25 mg/ml Pyridostigmine (5)	1.0×10^6	6.18 ± .09 (8)	6.21 ± .25 (8)
	2.5×10^6	6.49 ± .10 (8)	6.70 ± .18 (8)
50 mg/ml Pyridostigmine (5)	1.0×10^6	6.19 ± .09 (8)	6.21 ± .25 (8)
	2.5×10^6	6.. 4 ± .07 (8)	6.67 ± .22 (8)

* Concentration of peritoneal cells used

**Mean Log CPM of stimulated cultures ± standard deviation

Numbers in parentheses indicate number of rats used

TABLE 12: Chemiluminescence Response of Rat Peritoneal Exudate Cells Exposed to Pyridostigmine in vitro

Concentration of Pyridostigmine	Cell Concentration	
	$1 \times 10^{6/ml}$	$2.5 \times 10^{6}/ml$
0	6.34 ± .32*	6.58 ± .05
10^{-3} M	6.32 ± .29	6.48 ± .09
10^{-5} M	6.32 ± .28	6.52 ± .08
10^{-7} M	6.33 ± .28	6.53 ± .09
10^{-9} M	6.31 ± .30	6.55 ± .06

*Mean Log CPM of peritoneal cells stimulated with zymosan ± standard deviation
6 replicate cultures were assayed at each concentration of cells and drug

APPENDIX A

GENERAL ELISA PROTOCOL

A. Polyvinyl flat bottom plates. (i.e. Costar, Immulon, etc.)

B. PBS-Tween-20 Buffer pH 7.4 (PBS-T)

NaCl	16.0 g.	32.0 g.
KH_2PO_4 Monobasic	0.4	0.8
Na_2HPO_4 Dibasic	2.3	4.6
KCl	0.4	0.8
Tween 20	1.0 ml.	2.0 ml.
Merthiolate (Thimersol)	0.2	0.4
DPH_2O	*to 2 liters*	*to 4 liters*

pH to 7.4

C. Coating Buffer (Carbonate-bicarbonate pH 9.6). Store at room temperature for not more than 2 weeks.

1.59 g. Na_2CO_3

2.93 g. $NaHCO_3$

Bring to 1 liter with ddH_2O

D. Coating Plates with Antigen

1. Add 100μl. of appropriate dilution of antigen per well of 96-well plate. Make dilutions using Coating Buffer (See Sec. C)

2. Incubate overnight at room temperature.

3. Wash 3x with PBS-T

E. Blocking Non-specific Binding Sites

1. Add 200 μl. 3% BSA (wt./vol.) in PBS-T to each well.
2. Incubate 1 hour at room temperature*
3. Wash 3x with PBS-T.

*Plates can be frozen at this point with BSA in wells.

F. Addition of antisera

1. Make sample dilutions using PBS-T + 1% FBS
2. Add 50 μl. PBS-T to each well.
3. Add 50 μl. of sample dilution to be titrated to appropriate wells.
4. Make 2-fold dilutions
5. Incubate for 1 hour at 37°C.
6. Aspirate samples at this point.
7. Wash 3x with PBS-T.

G. Addition of Conjugate

1. Dilute conjugate to optimum concentration with PBS-T and 1% FBS.

   i.e. HRPO-Rb α Rat IgG 1:2000 (Horse Radish Peroxidase)

2. Add 100 μl. of diluted conjugate per well.
3. Incubate for 1 hour at 37°C
4. Wash 3x with PBS-T

H. Addition of Substrate

1. Mix: 31 ml. 0.1 M citric acid stock
   19 ml. 0.2 M Na_2HPO_4-$7H_2O$ stock

   Adjust to pH 4.0 using above stock solutions if necessary.

2. Add 11 mg. ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) to 50 ml. of mixed stock buffers.

3. Add 46 µl. of 30% hydrogen peroxide.

4. Add 100 µl. of substrate solution to each well.

5. Incubate for 15-45 minutes at 37°C.

6. Stop reaction with 20 µl. of 10% SDS.

I. Read plates at 410/450